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Regulation of Ethylene Evolution and Leaf Abscission by Auxin[1]

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Many physiological processes have shown analogous responses to both auxin and ethylene. These processes include root initiation, intumescence formation, epinasty (9), fruit ripening (3, 12), floral initiation in the pineapple (8, 19), breaking of bud dormancy (2, 4), stimulation of guttation (21, 22), and leaf abscission (1, 23).

Auxin applications can also stimulate ethylene evolution. Zimmerman and Wilcoxon (24) first noticed this phenomenon when tomato plants treated with IAA in lanolin produced an emanation which caused an epinastic response when confined with *Chenopodium album* and marigold. Later 2,4-dichlorophenoxyacetic acid (2,4-D) was found to stimulate ethylene evolution from pears (12) and cotton (15), and recently Morgan and Hall (16) have reported that ethylene production from cotton was stimulated by IAA.

Because ethylene and auxin have so many effects in common and since auxin has been found to stimulate ethylene evolution, ". . . some of the effects attributed to so-called growth substances might be due indirectly to the unsaturated hydrocarbon gas produced in the tissues" (24). This suggestion has been recently readvanced by others (15, 16).

Before an auxin-ethylene relationship can be established, however, it is necessary to show that auxin enhanced ethylene evolution occurs in a variety of plant material, and that ethylene evolution can be controlled by endogenous as well as exogenous levels of auxin. We will also demonstrate the importance of auxin regulated ethylene evolution on a physiological process, by investigating the abscission of bean petiole explants.

Materials and Methods

Plant material for the majority of the experiments consisted of *Phaseolus vulgaris* L. var. Red Kidney which was grown in soil in 10-cm pots. Etiolated plants were grown in the dark at 24 ± 2° and green seedlings were grown under identical temperatures with 1200 ft-c of fluorescent light given for 14 hours per day.

Ethylene evolved from experimental material was collected by placing the tissue sections in 43 ± 2 ml bottles (5 cm in diameter and 2.5 cm high) fitted with a neck to accommodate a 25 mm diameter rubber vaccine cap. The gas collection bottles were then sealed and incubated at 21°; etiolated tissue was placed in the dark and green tissue in 150 ft-c continuous light.

For abscission tests, explants were isolated consisting of the primary leaf abscission zone with 5 mm of tissue at the distal side and 5 mm of petiole at the proximal side. Six ml of agar were poured into the gas collection bottles and 10 explants were inserted into the agar so that 3 mm of the proximal ends were submerged. When desired, auxin was applied proximally by incorporation into the agar.

Two-milliliter gas samples were subsequently withdrawn from the bottles with a syringe that could be sealed with a petcock placed between the syringe barrel and the needle. These samples were injected into a F and M Scientific Corporation Model 720 gas chromatograph equipped with a Model 1609 flame ionization detector. Helium at 110 ml/minute flowed through a quarter inch, 60 cm activated alumina column. With the oven temperature at 45°, the analysis time was 1 minute. Authenticity of the ethylene peak was determined by cochromatography with standards (obtained from Olin Mathieson Company) and by use of mercuric perchlorate and NaCl as specific reagents for the absorption and release of ethylene (7).

Abscission data are expressed as mμl ethylene evolved per explant but can be converted approximately to parts per million by dividing by 3.4 or expressed as mμl/mg dry weight by dividing by 3.1 and then making the appropriate changes of units.

The area of the ethylene peak was determined by a disc chart integrator. Sensitivity of the chromatograph permitted the determination of 0.05 mμl ethylene per ml and the ethylene content of each sample could be determined with a precision of 5%.

IAA, β-(indole-3)-propionic acid (IPA), γ-(indole-3)-n-butyric acid (IBA), α-naphthaleneacetic acid (NAA), and gibberellic acid K salt (GA) were obtained from Calbiochem Corporation; 11.8% 2-chloroethyltrimethylammonium chloride (CCC) from Cyanamid International Company; 5% N-dimethylamine succinamic acid (B995) from U. S. Rubber Company, Naugatuck Chemical Division; and 2,4-dichlorobenzyltributylphosphonium chloride (Phosphon), from Virginia Carolina Chemical Corporation.

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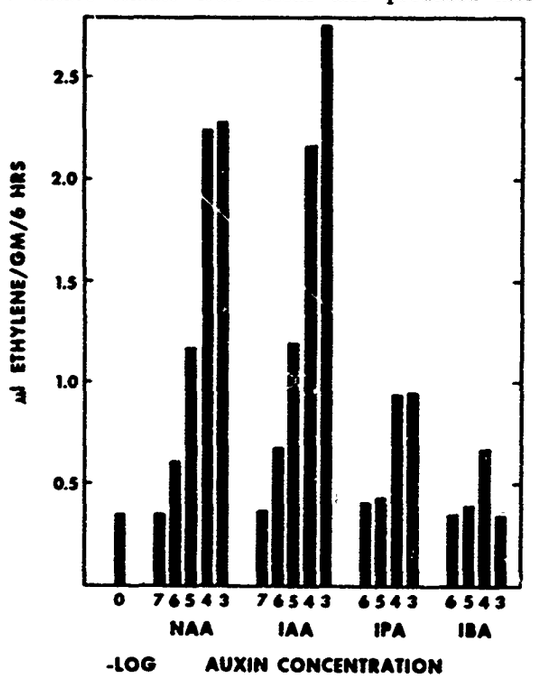
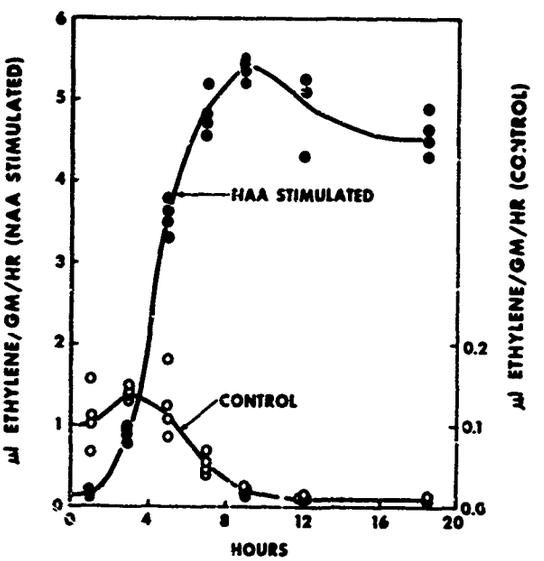
Results

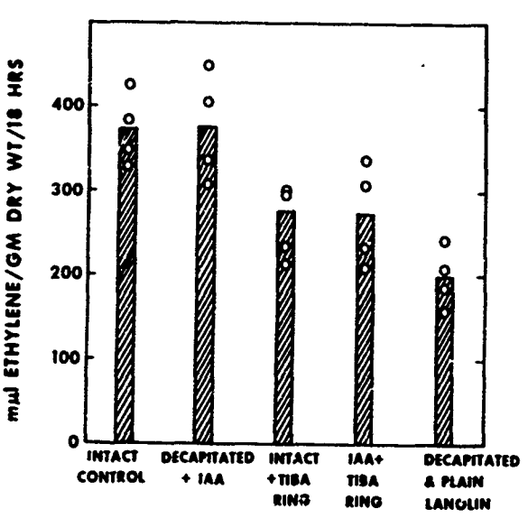
External Regulation of Auxin Levels. In order to demonstrate the ubiquity of auxin-induced ethylene evolution, parts of a wide range of plants were harvested and placed either on plain agar or agar containing 5×10^{-4} M NAA. The representative data in table I indicate that ethylene evolution from the vegetative organs of *Zea mays* L., *Phaseolus vulgaris* L. var. Red Kidney, and *Lycopersicum esculentum* Mill. is markedly increased by NAA. Similar results were obtained from *Zebrina pendula* Schnizl., *Coleus blumei* Benth., *Pisum sativum* L., *Manihot ultissima* Pohl., *Nicotiana tabacum* L., and *Coffea arabica* L. Immature tomato fruit tissue also produced more ethylene after auxin treatments, but ripening fruits showed the opposite response. Ethylene evolution from mature fruits of apple (*Malus pumula* Mill.) and pear (*Pyrus communis* L.) was also inhibited by NAA.

The Red Kidney bean was chosen for the remainder of the experiments. This was because these plants are easy to grow, they are a convenient material to demonstrate tropistic responses, and they could be related to earlier work on the abscission of petiole explants (20).

The effect of IAA, NAA, IPA, and IBA on ethylene production by week-old etiolated bean hypocotyls is shown in figure 1. Four 5 mm sections were cut from just below the cotyledonary node of 4







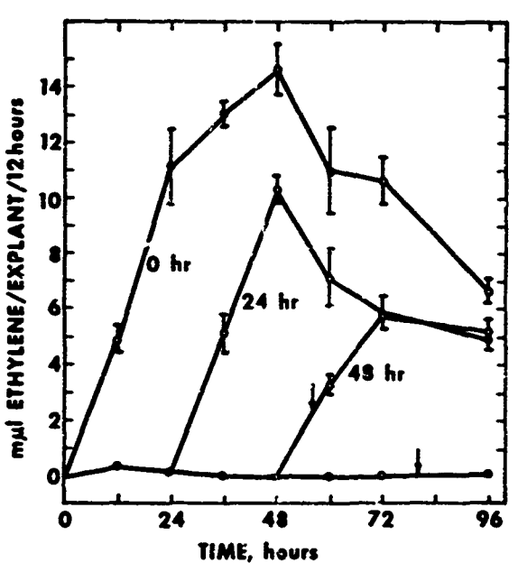


Table I. *Naphthaleneacetic Acid Stimulated Ethylene Evolution from Various Plant Species*
Leaf experiments were performed by floating ten 1 cm disks cut from young expanded leaves on solutions containing 5×10^{-4} M NAA. Other types of tissue were placed on 1 % agar with and without 5×10^{-4} M NAA. All were incubated at 21° for 18 hours; green tissue was exposed to 150 ft-c fluorescent light and root tissue stored in the dark.

Species	Plant part	mμl ethylene/g 18 hr		Remarks
		Control	5×10^{-4} M NAA	
Zea mays L.	root	750	1,400	14 days old
Zea mays L.	leaf blade	280	1,400	
Phaseolus vulgaris L.	root	930	1,800	7 days old
Phaseolus vulgaris L.	stem	230	2,400	
Phaseolus vulgaris L.	leaf blade	430	10,000	
Lycopersicum esculentum Mill.	root	4,600	6,980	mature plant
Lycopersicum esculentum Mill.	stem	220	1,400	
Lycopersicum esculentum Mill.	petiole	2,600	5,600	
Lycopersicum esculentum Mill.	leaf blade	490	1,500	
Lycopersicum esculentum Mill.	fruit	1,600	2,200	immature 11 mm diam.
Lycopersicum esculentum Mill.	fruit	3,800	2,300	pink stage

plants and placed either on plain agar or agar containing the growth regulating compounds. Earlier experiments established that 5 mm sections from this region of the hypocotyl showed the most rapid response to auxin. The material was incubated in the dark at 21° for 8 hours before the evolved ethylene was measured.

The weaker auxin analogues, IPA and IBA had a smaller stimulatory effect on ethylene evolution than the parent compound IAA. Since IAA and NAA had approximately similar effects on ethylene evolution, NAA was used in other experiments so as to minimize the destructive effects of enzymes released from cut surfaces.

Observations on the evolution of ethylene over time were carried out by placing the previously described 5 mm hypocotyl sections on plain agar or agar with 5×10^{-4} M NAA. Two-milliliter samples were then withdrawn every 2 hours, at which time the flasks were vented to the atmosphere and resealed. As seen in figure 2, about a 2-hour lag period was required before the auxin-treated tissue showed the characteristic increase in ethylene evolution. This was followed by an increasing rate of gas evolution which reached a maximum about 8 hours after the start of the experiments. The rate of ethylene evolution from control sections peaked between 2 to 4 hours and then rapidly declined.

Internal Regulation of Auxin Levels. According to Cholodny and Went, tropistic responses are due to an asymmetric distribution of auxin across a stimulated tissue. Since auxin stimulates ethylene evolution from bean hypocotyls, it is possible that the auxin gradient caused by a tropistic response would in turn give an ethylene gradient. For example, a geotropically stimulated bean hypocotyl will have more auxin on the lower than the upper side. If endogenous auxin controls ethylene evolution, then the lower side should evolve more ethylene than the upper side. The same argument would apply to a phototropic response in that the dark side might evolve more ethylene than the light side.

A 100-w microscope projector (American Optical Company Model 370) connected to a variac set at 100 v was used for illumination in the phototropism experiments. The light intensity at the plants was between 20 and 25 ft-c. After the seedlings responded by bending towards the light (5 hr), the curved portion of the hypocotyl was split by a razor blade into a dark and light side and placed into 2 gas collection bottles. Seven hypocotyl sections were placed into each bottle which was then incubated for 18 hours before withdrawing gas samples. Seedlings illuminated from above were used as controls. An experiment consisted of 5 pairs of bottles and the experiment was repeated 3 times. As shown by the representative experiment in table II, the light side had 43.7 % of the total ethylene evolving capacity of the curved hypocotyls. In vertically illuminated plants ethylene evolution was equally distributed between the 2 sides.

Geotropism experiments were performed by laying plants horizontally for 2 hours so that a visible growth response occurred. Curved portions of the hypocotyl were harvested and split into upper and lower sides, 7 plants being distributed between a set of bottles which were incubated similarly to phototropic experiments. Vertical plants were used as con-

FIG. 1 (*upper left*). Effect of auxin and auxin analogues on ethylene evolution from etiolated bean hypocotyls. (See text for details.)

FIG. 2 (*upper right*). Time course of auxin stimulated ethylene evolution from etiolated bean hypocotyls. (See text for details.)

FIG. 3 (*lower left*). Effect of decapitation and TIBA on ethylene evolution from week old etiolated bean seedlings. IAA, 1 mg/10 g lanolin, TIBA, 1 mg/1 g lanolin. (See text for details.)

FIG. 4 (*lower right*). Effect of NAA applications at various intervals after cutting on the production of ethylene by vented bean explants. Vertical arrows indicate hours to 50 % abscission. No abscission was observed for explants placed in NAA immediately or after 24 hours. The line through each point represents the standard error.

Table II. *Asymmetric Evolution of Ethylene from Etiolated Bean Hypocotyls*
Data are expressed as mμl ethylene evolved per g after 18 hours of incubation in the dark at 21 ± 1°.

	Phototropism				Geotropism			
	Vertical illumination*		Horizontal illumination*		Vertical plants		Horizontal plants**	
Side	A	B	Light	Dark	A	B	Upper	Lower
No. 1	179	204	130	166	176	177	150	182
2	222	224	154	189	205	191	122	167
3	274	235	161	210	150	149	137	162
4	187	217	137	224	154	141	113	128
5	199	204	210	233	172	165	160	195
6	...	...	...	...	153	161	118	133
Mean	212	217	158	204	168	164	133	161
% of total*** activity:	A = 49.2 ± 3.0		Light = 43.7 ± 3.8		A = 50.7 ± 1.7		Upper = 45.4 ± 1.8	
	P > 0.5		0.05		> 0.5		0.05	

* 20 to 25 ft-c for 5 hours.
** Placed horizontally for 2 hours.
*** Plus and minus standard deviation.

trols. An experiment consisted of 5 or 6 pairs of bottles and the experiment was repeated 6 times with consistent results. In a representative experiment (table II) the upper sides of geotropically stimulated seedlings produced 45.4 % of the total ethylene while the ethylene evolution from control plants was distributed equally between both sides.

The results of these experiments indicate that the asymmetric auxin distribution caused by tropistic stimuli results in a similar distribution of ethylene evolution and support the idea that internal auxin supply controls to some extent the rate of ethylene evolution.

It is generally accepted that auxin production occurs in the apex of plants and is transported away from its site of production in a polar manner. Decapitation or the inhibition of auxin transport by 2,3,5-triiodobenzoic acid (TIBA) (18), then, should decrease the auxin content of the subtending tissue. If endogenous auxin controls ethylene production, removal of the auxin supply by decapitation or TIBA ringing should decrease ethylene evolution from the hypocotyl tissue. Conversely, it should be possible to restore the original rate of ethylene evolution by application of lanolin containing an appropriate concentration of IAA.

To test the above proposition, 6 pots each containing 5 etiolated bean seedlings (1 week old) were treated in the following manner. Cotyledons were first removed from all plants (preliminary experiments indicated that cotyledons had no effect on ethylene evolution from the hypocotyls). One group of plants was left intact while others were decapitated by cutting just below the primary leaf node. The cut surface was covered either with plain lanolin or IAA lanolin (1 mg/10 g lanolin). This concentration of auxin was found to replace the apex for the ability of subtending tissue to evolve ethylene. Where specified, TIBA (1 mg/ g lanolin) was applied as a lanolin ring around the cotyledonary node. Eight hours later, 6 cm of hypocotyl were harvested from each plant after discarding all apical tissue by an incision 2 mm below the cotyledonary node. The hypocotyl segments were cut in half, placed in bottles, and incubated in the dark for 18 hours before the ethylene was measured.

The results of a representative experiment are shown in figure 3. The highest amounts of ethylene came from intact plants or decapitated plants treated with IAA. When the auxin source was removed by decapitation, smaller amounts of ethylene were detected. Similarly, restricting the polar transport of auxin in both intact or IAA-treated plants with TIBA markedly decreased the ethylene evolution of the hypocotyl sections.

Another method of varying the internal concentration of auxin may lie in the action of various growth retardants (11, 14) and GA (13, 17). Ten ml of growth retardants, CCC, B995, and Phosphon, were applied to 7-day-old green bean plants as a soil drench. Each treatment consisted of 7 pots with 4 plants in each and these were watered to leach the compounds into the soil. After 80 hours, stem tissue between the cotyledonary node and primary leaf node (first internode) was excised, measured, and incubated in gas collection bottles for 18 hours. The data in table III indicate that the compounds tested

Table III. *Inhibition of Ethylene Evolution from Phaseolus vulgaris by Growth Retardants*
First internode harvested 80 hours after treatment.

Treatment*	Length first internode mm**	mμl ethylene/ g 18 hr**
Control	46 ± 13	365 ± 44
10^{-2} M Phosfon	22 ± 8	142 ± 62
10^{-1} M CCC	17 ± 4	128 ± 12
10^{-1} M B995	23 ± 3	111 ± 24

* Ten ml of each solution applied as a soil drench per pot of week old seedlings.
** Mean ± standard deviation.

inhibited growth to one-third that of the controls and inhibited ethylene evolution by about one-third.

GA was applied by spraying week-old green bean plants (7 pots with 4 plants in each) until runoff and harvesting the first internode after 48 hours. Table IV shows that 10^{-3} M GA markedly stimulated ethylene evolution as well as elongation. This experiment was repeated 3 times with consistent results.

Relationship between Ethylene and Leaf Abscission. Since ethylene evolution appears to be accelerated by auxin and it is already known that the gas is a potent abscission stimulant, e.g. (23), attempts were then made to correlate the presence of ethylene with auxin-induced abscission promotion. Rubinstein and Leopold (20) have shown that bean leaf abscission could be separated into 2 stages, an initial stage when abscission was inhibited by NAA and a later stage when NAA stimulated abscission. The effect of NAA on ethylene production during these 2 stages was investigated using gas collection bottles that were vented every 12 hours (fig 4). If explants were placed in NAA at either 0 or 24 hours after cutting, abscission was inhibited and large amounts of ethylene were evolved. Insertion of explants into NAA 48 hours after cutting stimulated abscission, though less ethylene was detected as compared to those placed in NAA immediately. Similar results were obtained with IAA. One must conclude from these data that if ethylene is directly involved in accelerating explant abscission, its stimulatory effect is counteracted by the presence of NAA which retains the explants in the first abscission stage (20).

Attempts were then made to determine the effect of ethylene on each of the 2 abscission stages. This was done by injecting known quantities of the gas into gas collection bottles either immediately after placement of the explants or at 48 hours after placement; all treatments were vented at 12-hour intervals. The results in table V indicate that a 12-hour exposure of 0.34 to 3.40 mμl ethylene per explant was ineffective in stimulating abscission if applied immediately. When, however, the explants were exposed to the same concentrations of the gas at 48 to 60 hours after cutting, 50 % abscission occurred 20 hours sooner than the controls. These data suggest that ethylene, like amino acids (20), stimulates abscission only during the second stage.

If the ethylene produced is an important factor in the abscission process, then removal of the gas by flushing should decrease the rate of abscission. Because ethylene is formed within the explant, however, and there is a diffusion lag before it reaches the external gas phase, a flushing experiment cannot completely remove the gas from the site of action. Even so, any decrease in the time for 50 % abscission caused by aerating the explants would be an indication that ethylene is causally related to abscission.

To determine whether aeration could decrease abscission rates, stage 2 explants (explants left on plain agar for 48 hr) were placed on NAA agar with or without aeration (approximately 2 liter/min) and on plain agar with or without aeration. A 1.2 parts per million ethylene in air flush was used as a control to insure that the aerating treatment itself had no effect.

Table IV. *Gibberellin Induced Stimulation of Ethylene Evolution from the First Internode of Phaseolus vulgaris*

First internode harvested 48 hours after treatment.

Treatment*	Length first internode mm**	mμl ethylene/ g 18 hr**
Control	37 ± 17	275 ± 55
10^{-3} M GA	52 ± 11	540 ± 81

* Applied as spray until runoff to week old seedlings.
** Mean ± standard deviation.

Table V. *Hours to 50 % Abscission of Bean Explants after Ethylene Exposures at 0 or 48 Hours*

Ethylene mμl/explant	Time of exposure (hr)* 0 to 12	48 to 60
0.0	80 ± 3	80 ± 3
0.34	78 ± 5	58 ± 2
1.70	79 ± 7	56 ± 2
3.40	78 ± 3	55 ± 2

* Data represent hours to 50 % abscission with their standard errors.

By using hours to 50 % abscission as a criterion, it can be seen from figure 5 that sealed explants in NAA abscised first, explants flushed with 1.2 parts per million ethylene second, aerated explants in NAA third, control explants in sealed bottles fourth, and aerated controls abscised last. The data indicate that

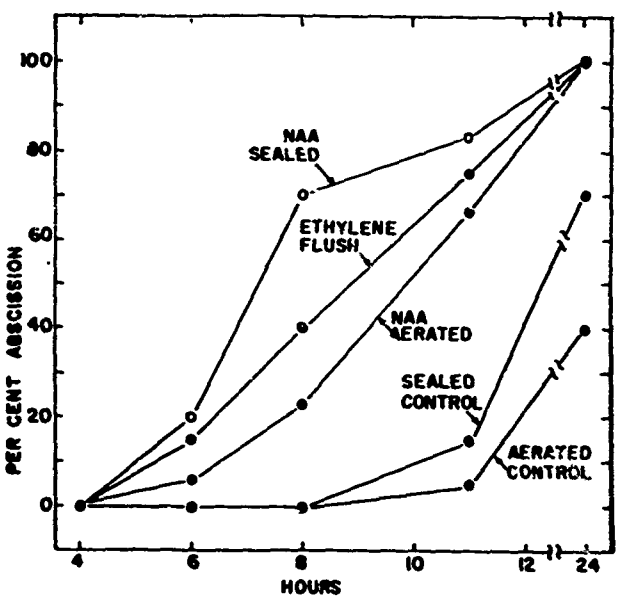


FIG. 5. Time curves illustrating abscission rates of bean petiole explants after various treatments to modify their atmosphere. Explants were placed in plain agar for 48 hours prior to start of the experiments. NAA was applied proximally at 5×10^{-4} M and the ethylene applied by continually flushing the explants with 1.2 parts per million.

removal of accumulated ethylene from the explants tended to delay the time of abscission. This experiment was repeated 5 times with almost identical results.

Discussion

Our work has centered on determining what relationships, if any, exist between auxins and ethylene production. Earlier studies by Zimmerman and Wilcoxon (24) and subsequent reports by others (12, 15, 16) have shown that ethylene evolution may be stimulated by exogenously supplied auxin. This phenomenon was confirmed here with the added observation that the weaker analogues of IAA, i.e., IPA and IBA, are less active as ethylene stimulators. However, the fact that auxin stimulates ethylene evolution does not make the presence of auxin a requirement for the production of this gas.

A wide range of plants and plant parts were treated with NAA to see if a stimulation of ethylene evolution was common to all. Every plant part tested gave off measurable amounts of ethylene and all vegetative parts showed marked increases in ethylene production after auxin treatments. Green tomato fruits also showed increases in ethylene after exposures to auxin, but NAA caused a decrease of ethylene evolution from mature fruits.

Next we attempted to show that ethylene evolution may be controlled by endogenous auxin levels. The Cholodny-Went theory for plant tropisms has been recently reexamined by Briggs (5) for phototropism in corn coleoptiles and by Gillespie and Thimann (10) for geotropism of *Avena* coleoptiles. Both papers agreed that the bending movements are due to a lateral redistribution of auxin to the side which grows most rapidly. We then exposed bean stems to geotropic and phototropic stimuli so as to induce an endogenous auxin gradient. When the stems were split longitudinally, it was found that the side which should contain more auxin than its opposite half also produced more ethylene.

The techniques of reducing endogenous auxin by decapitation or TIBA applications and it restoration by IAA applications are well documented. In these studies, the reduction of endogenous auxin resulted in simultaneous reductions in ethylene evolution, and the normal rate of ethylene production could be restored with IAA.

Another method of regulating internal auxin concentrations is based on Halevy's (11) observation that the growth retardants, Phosfon, CCC, and B995, induced greater amounts of extractable IAA oxidase and peroxidase in cucumber seedlings and a corresponding decrease in endogenous auxin. Kuraishi and Muir (14) reported that apices of pea plants whose growth was retarded by CCC had only one-seventh as much diffusable auxin as found in normal plants. In addition, stem sections of CCC-treated peas did not respond to GA but did respond to IAA. Experiments here show that treatment of bean plants with Phosfon, CCC, and B995 resulted in marked inhibitions of ethylene evolution and thus might be correlated to the hypothetical decrease in auxin.

In contrast to the action of growth retardants, GA may actually increase auxin levels since decreased IAA oxidase activity is observed after GA treatments (11). Kuraishi and Muir (13) have presented evidence that dwarf pea, normal pea, and sunflower yielded 3, 2, and 10 times more auxin respectively than untreated plants after GA application, and Nitsch (17) reported increases in extractable auxin after GA applications to *Rhus typhinia*. Accordingly, then, a further correlation exists between endogenous auxin levels and ethylene evolution, since applications of GA to bean plants markedly stimulated the rate of ethylene production.

The fact that stimulations of ethylene production occur concommitantly with auxin applications led to investigations concerning the NAA promotions of leaf abscission. Two stages of explant abscission have been proposed on the basis of NAA inhibitions of abscission when applied immediately and abscission stimulations when the auxin is applied later (20). It has been shown here that NAA stimulates ethylene evolution during both of these stages. The amount of ethylene present during the second stage was 3.5 mμl for explants inserted in NAA after 48 hours and since this concentration of ethylene has the ability to induce abscission if added during stage 2 (table V), we feel that the presence of ethylene may explain the induction of explant abscission after auxin applications.

Before an ethylene stimulation can occur, however, the explants must be in the second stage as measured by the increased rate of abscission upon contact with NAA. Thus, even though immediate applications of NAA stimulate the production of high amounts of ethylene, the gas is unable to promote abscission because the NAA simultaneously retains the explants in the first abscission stage.

To critically investigate the relationship between ethylene and abscission, it is not merely sufficient to show that ethylene is always produced prior to abscission. It should also be possible to demonstrate that abscission can be inhibited when the level of ethylene present at the active site is reduced. As yet it is not feasible to prevent ethylene evolution by means of an inhibitor because no substance is known which will inhibit only the production of ethylene and not equally retard other vital metabolic processes (6).

Another approach has been to aerate abscission zones as rapidly as possible so that only a minimum of ethylene could accumulate. Hansen (12) reported that treatment of immature pears with 2,4-D resulted in increases in the rates of ethylene evolution and ripening. When the fruit was aerated, however, the acceleration of ripening by the 2,4-D was markedly decreased.

Utilizing this aeration technique, we observed small but consistent differences which suggested that the acceleration of abscission by NAA could be inhibited by the flushing treatment. The fact that aerated explants still abscised may indicate the failure